Current Biology 19, 583–589, April 14, 2009 ©2009 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2009.02.040

Report

# A Hypervariable Invertebrate Allodeterminant

Matthew L. Nicotra,[1,6] Anahid E. Powell,[1] Rafael D. Rosengarten,[2] Maria Moreno,[2] Jane Grimwood,[4] Fadi G. Lakkis,[5] Stephen L. Dellaporta,[2,*] and Leo W. Buss[1,3,*]

[1]Department of Ecology and Evolutionary Biology
[2]Department of Molecular, Cellular, and Developmental Biology
[3]Department of Geology and Geophysics
Yale University
New Haven, CT 06511
USA
[4]HudsonAlpha Genome Sequencing Center
HudsonAlpha Institute for Biotechnology
Huntsville, AL 35806
USA
[5]Thomas E. Starzl Transplantation Institute
Departments of Surgery and Immunology
University of Pittsburgh School of Medicine
Pittsburgh, PA 15261
USA

## Summary

**Colonial marine invertebrates, such as sponges, corals, bryozoans, and ascidians, often live in densely populated communities where they encounter other members of their species as they grow over their substratum. Such encounters typically lead to a natural histocompatibility response in which colonies either fuse to become a single, chimeric colony or reject and aggressively compete for space. These allorecognition phenomena mediate intraspecific competition [1–3], support allotypic diversity [4], control the level at which selection acts [5–8], and resemble allogeneic interactions in pregnancy and transplantation [9–12]. Despite the ubiquity of allorecognition in colonial phyla, however, its molecular basis has not been identified beyond what is currently known about histocompatibility in vertebrates and protochordates. We positionally cloned an allorecognition gene by using inbred strains of the cnidarian, *Hydractinia symbiolongicarpus*, which is a model system for the study of invertebrate allorecognition. The gene identified encodes a putative transmembrane receptor expressed in all tissues capable of allorecognition that is highly polymorphic and predicts allorecognition responses in laboratory and field-derived strains. This study reveals that a previously undescribed hypervariable molecule bearing three extracellular domains with greatest sequence similarity to the immunoglobulin superfamily is an allodeterminant in a lower metazoan.**

*Correspondence: stephen.dellaporta@yale.edu (S.L.D.), leo.buss@yale.edu (L.W.B.)
[6]Present address: Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA 02138, USA

## Results and Discussion

*Hydractinia* colonies consist of polyps specialized for feeding, reproduction, or defense, which bud from the mat, a sheet of two ectodermal cell layers that encase a network of endodermal gastrovascular canals (Figure 1A). Colonies grow by expanding the leading edge of the mat or by elongating stolons, ectodermally covered extensions of gastrovascular canals. When two *Hydractinia* colonies grow into contact, they undergo the fusion-rejection response. Fusion is characterized by ectodermal cell adhesion and establishment of a continuous gastrovascular system [13, 14] (Figures 1B and 1C). In contrast, rejection is characterized by failure of ectodermal cells to adhere and extensive recruitment of nematocytes to the contact site [13, 14]. Nematocytes are a cnidarian-specific cell type that contains nematocysts, harpoon-like organelles used for feeding and defense. Nematocytes at the contact site do not initially discharge their nematocysts but instead assemble at the colony margin (Figure 1D) and simultaneously fire after contact, destroying the foreign tissue. Additional nematocytes subsequently migrate to the contact zone and discharge their nematocysts once they are oriented toward the opposing colony [14]. During rejection responses, stolons frequently become hyperplastic, swelling with nematocytes, rising off the substrate, and growing over the opposing colony (Figures 1E and 1F) [13–15].

Investigation of the genetics of allorecognition in *Hydractinia* began in the 1950s, when Hauenschild [16, 17] performed systematic breeding experiments in which he created two $F_2$ populations derived from two separate pairs of wild-type colonies. He interpreted the results of these crosses via a single-locus model of inheritance, but noted that this model did not explain a small proportion of fusibility results in the $F_1$ and $F_2$ generations. Later investigators similarly reported fusibility results inconsistent with simple, single-locus Mendelian inheritance in $F_1$ and $F_2$ populations derived from wild-type colonies [18]. Subsequently, our lab established inbred lines of *Hydractinia* and demonstrated that, in these lines, allorecognition segregated as a pair of tightly linked loci, *alr1* and *alr2*, which mapped to a 1.7 cM chromosomal interval [19–21] (Figure 2A). Within our inbred strains, two alleles (*f* and *r*) segregate at each locus. Colonies sharing at least one allele at both loci fuse, whereas colonies sharing no alleles at either locus reject. Colonies sharing alleles at only one locus undergo transitory fusion, which can be of two types [20, 21]. If colonies share at least one allele at *alr2* and none at *alr1*, they initially fuse, but develop a gray band across the contact zone 1–3 days after fusion, their gastrovascular canals become occluded, and they permanently separate within 1–3 days (type I transitory fusion) [20, 21]. In contrast, colonies sharing at least one allele at *alr1* and none at *alr2* fuse normally for 1–4 days, then display cycles of separation and refusion that continue indefinitely (type II transitory fusion) [21]. Thus, *alr1* and *alr2* jointly distinguish self from non-self.

### Positional Cloning and Identification of an *alr2* Candidate Gene

We positionally cloned *alr2* by using a tightly linked molecular marker (marker 174, Figure 2B) [20]. Bacterial artificial

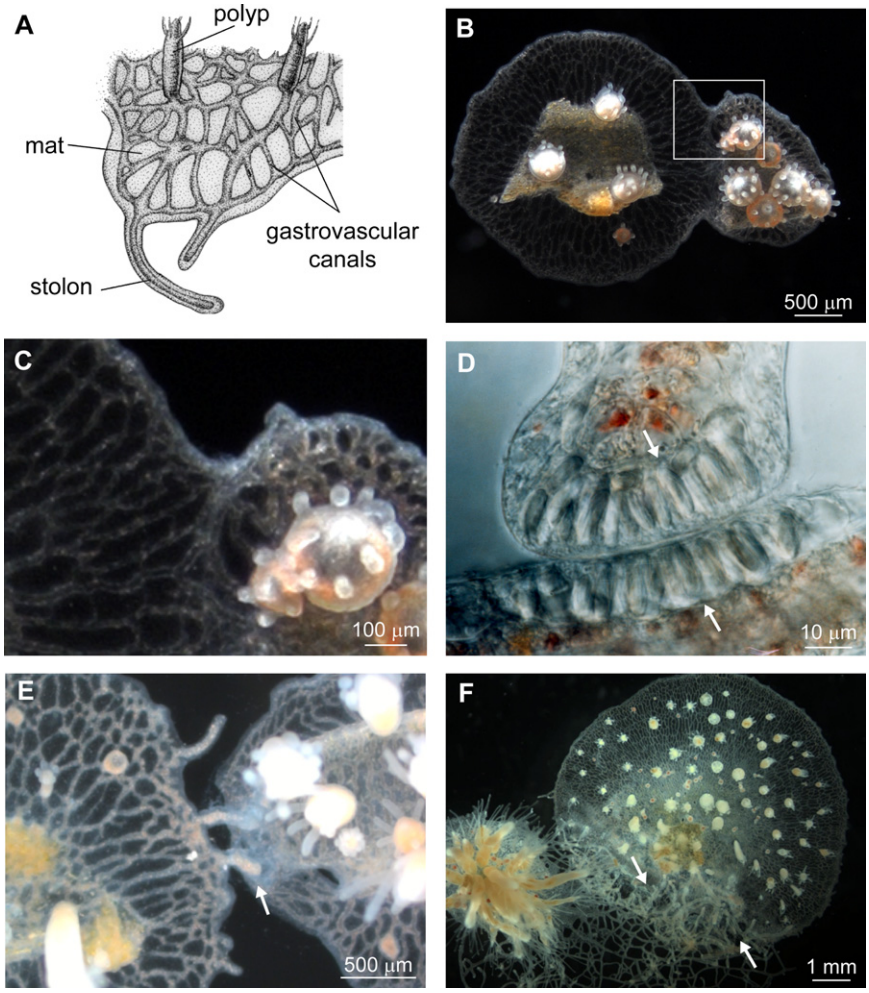


Figure 1. Allorecognition in *Hydractinia symbiolongicarpus*

(A) Morphology of a *Hydractinia* colony. Modified from [37].

(B) Fusion between two *Hydractinia* colonies.

(C) Magnification of the boxed area in (B).

(B and C) Reprinted with permission from [21].

(D) Magnification of a stolon tip and stolon flank from rejecting colonies, showing recruitment of nematocytes (arrowheads) to the contact point. From [14], reprinted with permission of John Wiley & Sons, Inc. © 1989.

(E) Early rejection, showing production of hyperplastic stolons (arrowhead).

(F) Late stage rejection, showing proliferation of hyperplastic stolons (arrowheads). Reprinted with permission from [26].

(D), (E), and (F) show different colonies.

chromosome (BAC) and fosmid genomic libraries were constructed from a laboratory strain homozygous for the *f* allele at both *alr1* and *alr2* (colony 833-8). We used previously identified recombinants [20, 21] to define the proximal and distal limits of the *alr2* locus with six recombination breakpoints, localized to two ~15 kb regions at each end of the contig (X's, Figure 2B).

The minimum tiling path over the *alr2* region was sequenced and analyzed for putative coding sequences (CDS), of which we identified nine (Figure 2B and Table S1 available online). For each CDS, we obtained a full-length cDNA sequence and analyzed expression via RT-PCR. Similarity searches comparing each putative CDS against protein and conserved domain databases identified these genes as likely homologs of a *Nematostella* malate dehydrogenase (CDS1), a mosquito hypothetical protein bearing ankyrin repeats and a KH RNA-binding domain (CDS2), bovine actin-related protein 8 (CDS3), chicken ATP synthase mitochondrial F1 complex assembly factor 2 (CDS4), and honeybee alpha-1,6-fucsosyltransferase (CDS8). The top BLAST hit for CDS7 was to a protein of unknown function from the nematode *Brugia malayi*, whereas CDS9 was not similar to any known protein. The remaining two sequences, CDS5 and CDS6, were partial duplications of CDS7 (Figure S1). Although cDNA products for CDS5 and CDS6 were amplified via 5′ RACE, 3′ RACE experiments with the same cDNA pools successfully employed in all other RACE experiments failed repeatedly, suggesting that these sequences lacked polyadenylation. In addition, CDS5 included a frame-shift mutation that inserted a stop codon in its third exon. These data led us to conclude that CDS5 and CDS6 were likely pseudogenes arising from tandem duplication of the CDS7 genomic region, and we therefore designated them CDS5P and CDS6P.

Initial evaluation of *alr2* candidate genes used two criteria. The fact that allorecognition responses require cell-cell contact and involve tissue adhesion [13, 14] suggests that *alr2* is a membrane-associated protein. In addition, an *alr2* candidate must display substantial polymorphism between histo-incompatible inbred strains, i.e., between *f* and *r* alleles. Only CDS7 proved to both encode a transmembrane protein and possess a highly polymorphic extracellular domain (Table S1). CDS7 was predicted to contain 9 exons with a 2.3 kb full-length cDNA encoding 672 amino acids (Figure 2D). The protein was a predicted type I transmembrane protein with an 18 amino acid (aa) signal peptide followed by a 411 aa extracellular region consisting of three domains, a single 23 aa transmembrane helix, and a 220 aa cytoplasmic domain. The cytoplasmic domain contained an endocytosis motif and potential phosphorylation sites for tyrosine kinase, protein kinase C, and casein kinase II. We also searched CDS7 for immunoreceptor tyrosine-based activation motifs (ITAMs) (Y-xx-I/L-$x_{(6-12)}$-Y-xx-L/I, where x is any amino acid) and immunoreceptor tyrosine-based inhibitory motifs (ITIMs) ((I/L/V/S)-x-Y-xx-I/V/L), signaling motifs frequently found in the cytoplasmic domains of vertebrate immune receptors [22]. Although we did not locate any canonical ITAMs or ITIMs, we did find a single ITIM-like motif (N-x-Y-xx-V) previously identified on members of the leukocyte immunoglobulin-like receptor (LIR) family [23] (Figure 2D). Alignment of CDS7-*f* and CDS7-*r* revealed 26 aa polymorphisms, of which 17 were located in the 114 aa region encoded by exon 2 (Figure 2D). This level of polymorphism was the highest of any expressed sequence in the *alr2* genomic region (Table S1). Southern blot hybridization was consistent with CDS7 being a single copy sequence in the *Hydractinia* genome (Figure S2).

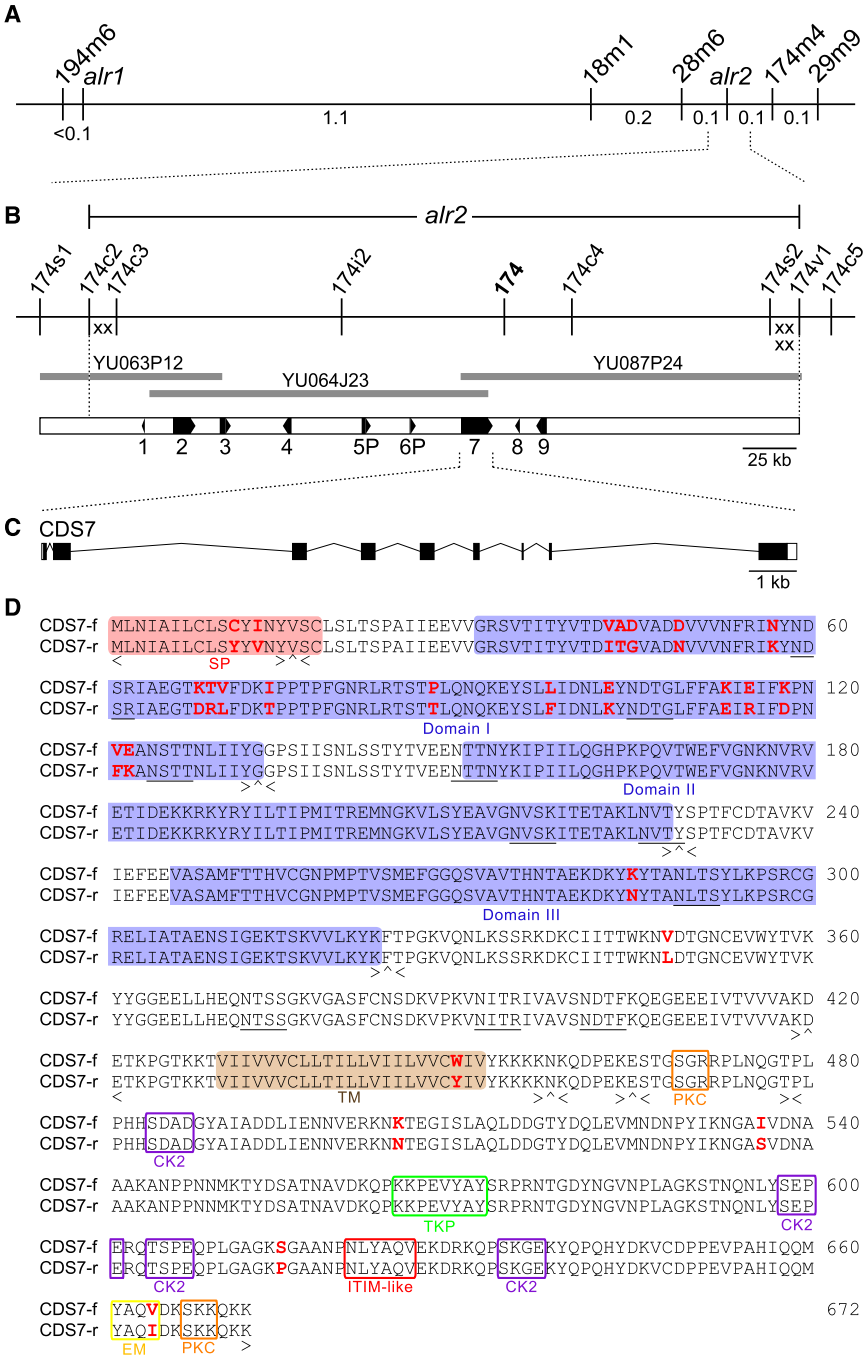


Figure 2. Positional Cloning of *alr2*

(A) Genetic map of the *Hydractinia* allorecognition complex, which contains two histocompatibility loci, *alr1* and *alr2*. 194m6, 18m1, 28m6, 174m4, and 29m9 are markers used to map the interval. Genetic distances are shown below the line in centimorgans.

(B) Physical map of the *alr2* genomic region. Markers are derived from fosmid or BAC sequences. Recombination breakpoints defining the *alr2* locus are shown as X's. BAC clones comprising the minimum tiling path across the *alr2* region are shown as heavy gray lines. Numbered boxes are expressed coding sequences.

(C) Genomic organization of CDS7. White boxes, black boxes, and bent lines indicate UTRs, exons, and introns, respectively.

(D) CDS7 amino acid sequence, showing alignment between the *f* and *r* alleles. SP, signal peptide; domains I–III, regions similar to Ig-like domains; TM, transmembrane domain; PKC, protein kinase C phosphorylation motif; TKP, tyrosine kinase phosphorylation motif; CK2, casein kinase II phosphorylation motif; EM, endocytosis motif; ITIM-like, ITIM-like motif. Potential N-glycosylation sites are underlined. Exon organization is denoted below the alignment. < and > indicate amino acids encoded by the last complete codon of the left and right exon ends, respectively. ^ indicates codon spanning two exons.

BLASTP searches with CDS7 returned significant alignments to diverse proteins from the immunoglobulin superfamily. These alignments were exclusively between the extracellular portion of CDS7 encoded by exons 2, 3, and 4 (hereafter domains I, II, and III; Figure 2D) and full or partial immunoglobulin-like (Ig-like) domains. Althuogh the most significant alignment was to a hypothetical *Brugia* protein ($2 \times 10^{-6}$, 23% identity), most BLAST hits were to members of the IgLON family of neural cell adhesion molecules with similar *e*-values and % identities (see Supplemental Results and Discussion). To further explore the similarity between CDS7 and the Ig superfamily, we searched conserved domain databases and performed similarity searches based on tertiary structure, which should be sensitive to distant homologies. These methods consistently predicted Ig-like folds for domains I–III, with domain I most similar to V-set domains, and domains II–III most similar to I-set domains (Table S2). Multiple sequence alignments between domains I–III and canonical I- and V-set domains showed the *Hydractinia* domains matched the common V/I-set frame residues [24] at many positions, although only domain II possessed the highly conserved tryptophan and all domains lacked the hyperconserved cysteines characteristic of most Ig-like domains (Figure S3 and Supplemental Results and Discussion). Together, these analyses show that domains I–III are most similar to Ig-like domains and suggest that CDS7 could be a novel member of the Ig superfamily, albeit a distinct one.

BLAST searches against the only sequenced hydrozoan genome, *Hydra magnipapillata*, returned a single significant alignment between CDS7 domains I–III and three Ig-like domains from a predicted titin-like molecule ($e = 0.009$, 24% identity, scaffold ID: NW_002165237). Similarly, BLAST searches against the genome of the starlet anemone, *Nematostella vectensis*, returned significant alignments between domains II and III and several putative Ig-superfamily members, with the top hit to a predicted protein similar to mammalian neural cell adhesion molecules ($e = 1 \times 10^{-5}$, 27% identity, accession: XP_001637446). We found no synteny between the *alr2* genomic region and the *Nematostella* genome, but

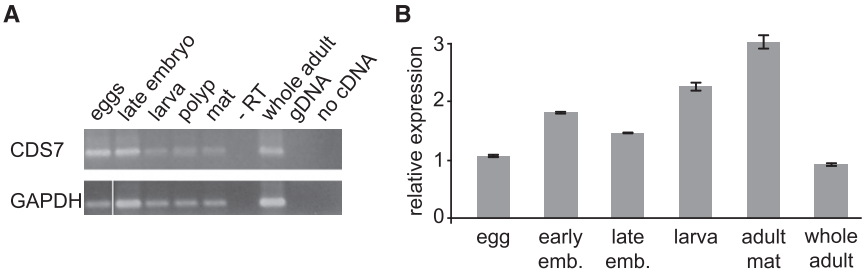


Figure 3. Expression of CDS7

(A) RT-PCR of cDNA isolated from different *Hydractinia* life stages and tissues. Top panel shows a 509 bp product amplified from exons 8–9 of CDS7. Bottom panel shows a 406 bp product amplified from GAPDH. –RT indicates control in which reverse transcriptase was omitted from cDNA synthesis.

(B) Real-time PCR of CDS7. Relative expression shown for eggs, 2- to 8-cell embryos (early blastula), 64-cell embryos (late blastula), planulae, adult mat tissue, and whole adult colonies (polyps + mat). Error bars indicate standard error for triplicates.

did detect two small tracks of synteny between the *alr2* interval and two different scaffolds in the *Hydra* genome assembly (Figure S4). Neither track of synteny included CDS7, and neither *Hydra* scaffold encoded any genes with immunoglobulin-like domains. TBLASTN searches with CDS7 against cnidarian ESTs did not return significant alignments. No significant similarity was detected between CDS7 and the FuHC and fester allorecognition proteins from the protochordate tunicate *Botryllus schlosseri* [10, 25].

### Expression of the *alr2* Candidate Gene

An *alr2* candidate gene should be expressed in all tissues capable of allorecognition. In *Hydractinia*, late larval, polyp, and mat tissues display allorecognition phenomena [13, 14], whereas blastulae and early stage larvae do not [26, 27]. CDS7 expression was assayed qualitatively by RT-PCR in cDNA pools representing five tissue types (eggs, 64-cell embryos, 2- to 3-day-old larvae, mat, and polyps) and was detectable in all tissues examined (Figure 3A). Quantitative RT-PCR showed that expression was highest in the adult mat tissue (Figure 3B). We suspect that the CDS7 expression we observed in early embryonic tissues reflects a need for allodeterminants to be deployed in earliest colony ontogeny, because *Hydractinia* colonies possess short-lived, non-feeding larvae that settle on hermit crab shells in a site-specific fashion and have a high probability of encountering conspecifics immediately post-metamorphosis [28].

### Polymorphism and Phenotype Prediction of the *alr2* Candidate Gene

To further characterize CDS7's role as an allodeterminant, we employed a stringent test similar to that used to identify the *FuHC* histocompatibility locus in *Botryllus* [10]. Because *Hydractinia* colonies must share at least one allele at either *alr1, alr2,* or both to avoid rejection, and both fusion and transitory fusion are exceedingly rare between field-collected colonies [18, 19, 29], these loci are predicted to be highly polymorphic [4]. Moreover, pairs of field-collected colonies that do not reject should share at least one allele at either *alr1* or *alr2*, which is an event unlikely to occur by chance [4].

We assessed CDS7 polymorphism by examining alleles from our two inbred strains and seven wild-type colonies for a total of 16 alleles. Domain I was highly variable (Figure 4A) and had an average of 31 pair-wise differences between alleles. Although 68/111 amino acid positions in domain I differed between at least two alleles, most residues that aligned to the conserved V/I-frame residues in canonical Ig-like domains were invariant (Figure 4B). We also analyzed CDS7 for evidence of positive selection by identifying codons at which the estimated rate of nonsynonymous mutation exceeded that of synonymous mutation. Allorecognition systems are expected to be under positive frequency-dependent selection, which favors rare alleles [4]. Site-wise analysis with the HyPhy Statistical package [30] identified six positively selected codons, all within domain I (Figure 4B and Table S3). Thus, CDS7 had both of the hallmarks of frequency-dependent selection—high allele number at low frequency [31] and positively selected sites.

To test whether CDS7 could predict allorecognition responses, we screened field-collected colonies for their ability to fuse to inbred colonies bearing either *f* (n = 497) or *r* (n = 508) alleles at *alr1* and *alr2*. Only two colonies (0.2%) failed to reject laboratory strains. Colony LH416 displayed type II transitory fusion against the *f* tester colony, consistent with an *f*-like allele at *alr1*, and colony LH82 displayed type I transitory fusion against the *f* tester colony, consistent with an *f*-like allele at *alr2*. We sequenced full-length cDNAs for the two CDS7 alleles from LH82 (designated *a* and *b*), as well as the genomic regions encoding them. Predicted amino acid sequences of the *a* and *f* alleles differed at 41/672 sites, including 31/119 sites in domain I. In contrast, the *b* and *f* alleles were 100% identical over domain I and differed at only 7/672 sites overall (1 in the signal peptide, 1 in domain III, 1 in the remaining extracellular domain, and 4 in the cytoplasmic domain). A test cross between LH82 (CDS7-*a*/*b*) and a colony homozygous for *r* at both allorecognition loci demonstrated that the CDS7-*b* allele cosegregated with an ability to display transitory fusion against colonies bearing CDS7-*f* alleles (number of offspring in cross = 12; 5 CDS7-*a*/*r* offspring, all rejected homozygous *f* tester; 7 CDS7-*b*/*r* offspring, all displayed transitory fusion to homozygous *f* tester). Thus, the only wild-type colony with a phenotype suggesting it shared a common *alr2* allele with our *f* inbred line also carried a CDS7 allele 100% identical to the *f* allele over the hypervariable extracellular domain. More extensive analysis of the map between sequence variation and fusibility in natural populations is now underway.

Because our data indicated that CDS7 was an allodeterminant in our inbred lines, displayed extensive natural polymorphism, and predicted allorecognition responses between our inbred lines and field-collected colonies, we concluded it was *alr2*. Identification of this cnidarian histocompatibility gene creates an immediate opportunity to address several long-standing questions about invertebrate allorecognition, including the population genetic mechanisms maintaining variation, the role that chimerism plays in the distribution and abundance of colonial organisms, and the degree of conservation, if any, between allorecognition systems in colonial taxa. In addition, relationships have often been suggested between cnidarian and protochordate allorecognition systems or

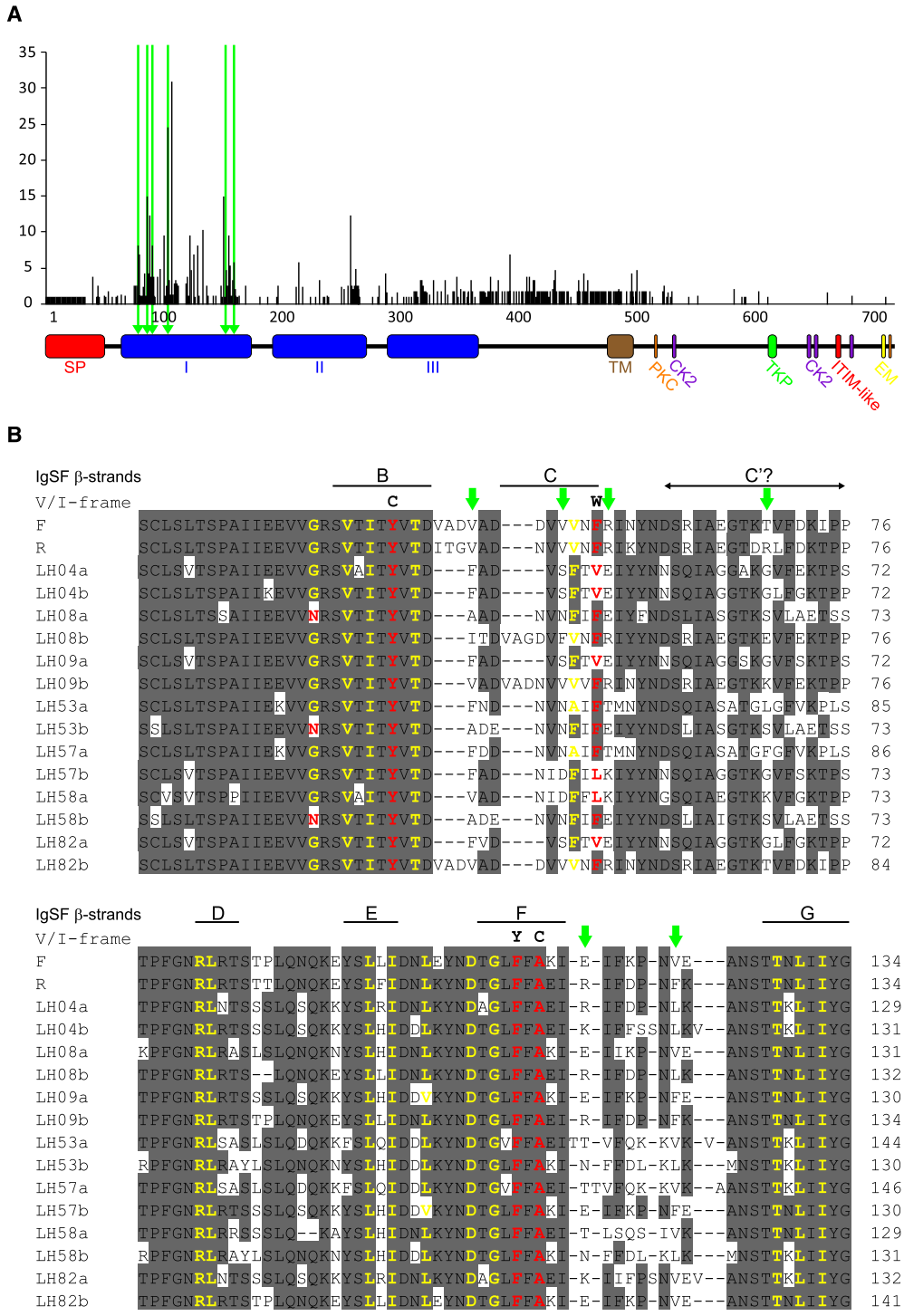


Figure 4. Variability of CDS7

(A) High levels of per-site variability suggest positive selection for diversity over CDS7. Variability was calculated as (number of different residues)/(frequency of the most common residue) − 1. Triangles and green bars indicate sites demonstrating significant departures from neutrality, suggesting positive selection. CDS7 domains labeled as in Figure 2.

(B) PRANKF [25] alignment of domain I from 16 CDS7 alleles. Eight or more consensus residues are shaded gray. Green arrowheads indicate positions under positive selection. Residues matching the conserved V/I-frame are in yellow, those that differ are in red, with V/I-frame consensus residues shown above. Beta-strand positions in canonical Ig-like domains relative to the putative V/I-frame residues are shown above the alignment.

between invertebrate allorecognition systems and elements of the vertebrate immune system, particularly the MHC. Although Ig-like domains are found in vertebrate immune molecules, the *Botryllus FuHC* gene, and potentially *alr2*, there appears to be no additional similarity between the known surface molecules in these three systems. Indeed, growing evidence suggests

that animals have evolved a variety of unique molecular mechanisms to distinguish self from non-self, including the MHC in vertebrates, VCBPs in protochordates [32], VLR immune molecules in jawless fish [33], FREP proteins in molluscs [34], and the FuHC in tunicates [10]. We can now add the *Hydractinia* allorecognition system to this diversity. It remains possible, however, that invertebrate and vertebrate histocompatibility systems share downstream signaling pathways. The presence of an ITIM-like motif in the cytoplasmic domain of *alr2* suggests that it could be phosphorylated by SH2-domain containing protein tyrosine phosphotases similar to those involved in inhibitory signaling in the vertebrate immune system. As additional molecular data become available for *Hydractinia* and other invertebrate allorecognition systems, we will finally be able to address these questions.

## Experimental Procedures

Detailed experimental procedures are provided in Supplemental Data, but are summarized here.

### Positional Cloning

Fosmid and BAC libraries were constructed with DNA from an inbred colony homozygous for the *f* allele at *alr1* and *alr2*. Clones isolated during the chromosome walk were assembled into contigs via restriction digest fingerprinting [35], with overlaps confirmed by PCR. The minimum tiling path of the *alr2* region was sequenced through the Community Sequencing Project of the Joint Genome Institute, US Department of Energy.

### Sequence Analysis and CDS Identification

Potential coding sequences were identified with a combination of BLAST similarity searches and ab initio gene prediction algorithms. Full-length cDNAs of *f* alleles were obtained by RACE and RT-PCR experiments with the GeneRacer Kit (Invitrogen, Carlsbad, CA).

### Expression Assays

First-strand cDNA pools were created from eggs, blastulae, larvae, polyp, and mat tissues via SuperScript III reverse transcriptase (Invitrogen) primed with the Oligo-dT primer supplied in the GeneRacer Kit. CDS7 expression was assessed qualitatively by RT-PCR by amplification of a 509 bp fragment spanning exons 6–9. As a control, a 406 bp region of GAPDH spanning the predicted stop codon was used. For real-time quantitative PCR, primers amplifying a 156 bp region of exons 8–9 of CDS7 or a 117 bp region of exons 4–5 of GAPDH were used.

### Polymorphism and Selection Analyses

For each candidate gene, a full-length cDNA sequence of the *f* alleles was obtained by RT-PCR. Sequences of *r* alleles were predicted by mapping the cDNA sequence of the *f* allele onto the genomic sequence of the *r* haplotype, which was obtained with a BAC library constructed from an inbred colony homozygous for the *r* allele at *alr1* and *alr2* and kindly provided by Luis Cadavid (National University of Columbia).

For CDS7, full-length cDNA sequences were obtained from the two inbred alleles plus seven wild-type colonies, for a total of 16 alleles. Predicted amino acid sequences were aligned with $PRANK_{+F}$ [36]. For positive selection analyses, the alignment was back-translated to generate a nucleic acid alignment. Site-wise maximum-likelihood analyses for positive selection were performed with the Datamonkey server, which runs the *HyPhy* software package [30]. We reported sites to be under positive selection if they received significant scores under two out of three different codon-based maximum likelihood methods (SLAC, REL, and FEL).

### Supplemental Data

Supplemental Data include Supplemental Results and Discussion, Supplemental Experimental Procedures, five figures, and five tables and can be found with this article online at http://www.current-biology.com/supplemental/S0960-9822(09)00750-7.

### Acknowledgments

We thank K. Altland, C. Glastris, E. Buss, S. Lubner, and T. Wu for technical assistance, L. Cadavid for providing BAC clones of the *r* haplotype, and A. Signorovitch, M. Flajnik, and L. Du Pasquier for discussion. Supported by NIH grant 1R21-AI066242 (F.G.L., S.L.D., and L.W.B.), NSF grant IOS-0818295 (L.W.B. and S.L.D.), the American Society of Nephrology (F.G.L.), and the Joint Genome Institute's Community Sequencing Program (L.W.B. and S.L.D). M.L.N. was supported by a US Department of Education GAANN Fellowship, A.E.P. was supported by NIH Training Grant T32-GM07499 in Genetics, and R.D.R. was supported by a National Science Foundation Graduate Research Fellowship. *alr2* sequences available in GenBank (EU219736, FJ207397–FJ207400, FJ207403–FJ207409, FJ617565–FJ617568).

Received: January 8, 2009
Revised: February 8, 2009
Accepted: February 9, 2009
Published online: March 19, 2009

### References

1. Francis, L. (1973). Intraspecific aggression and its effect on the distribution of *Anthopleura elegantissima* and some related sea anemones. Biol. Bull. *144*, 73–92.
2. Buss, L.W., and Grosberg, R.K. (1990). Morphogenetic basis for phenotypic differences in hydroid competitive behaviour. Nature *343*, 63–66.
3. Buss, L.W. (1990). Competition within and between encrusting clonal invertebrates. Trends Ecol. Evol. *5*, 352–356.
4. Grosberg, R.K. (1988). The evolution of allorecognition specificity in clonal invertebrates. Q. Rev. Biol. *63*, 377–412.
5. Stoner, D.S., Rinkevich, B., and Weissman, I.L. (1999). Heritable germ and somatic cell lineage competitions in chimeric colonial protochordates. Proc. Natl. Acad. Sci. USA *96*, 9148–9153.
6. Buss, L.W. (1982). Somatic cell parasitism and the evolution of somatic tissue compatibility. Proc. Natl. Acad. Sci. USA *79*, 5337–5341.
7. Buss, L.W. (1987). The Evolution of Individuality (Princeton, NJ: Princeton University Press).
8. Laird, D.J., De Tomaso, A.W., and Weissman, I.L. (2005). Stem cells are units of natural selection in a colonial ascidian. Cell *123*, 1351–1360.
9. Burnet, F.M. (1971). "Self-recognition" in colonial marine forms and flowering plants in relation to the evolution of immunity. Nature *232*, 230–235.
10. De Tomaso, A.W., Nyholm, S.V., Palmeri, K.J., Ishizuka, K.J., Ludington, W.B., Mitchel, K., and Weissman, I.L. (2005). Isolation and characterization of a protochordate histocompatibility locus. Nature *438*, 454–459.
11. Litman, G.W., Cannon, J.P., and Dishaw, L.J. (2005). Reconstructing immune phylogeny: new perspectives. Nat. Rev. Immunol. *5*, 866–879.
12. Laird, D.J., De Tomaso, A.W., Cooper, M.D., and Weissman, I.L. (2000). 50 million years of chordate evolution: seeking the origins of adaptive immunity. Proc. Natl. Acad. Sci. USA *97*, 6924–6926.
13. Buss, L.W., McFadden, C.S., and Keene, D.R. (1984). Biology of hydractiniid hydroids. 2. Histocompatibility effector system competitive mechanism mediated by nematocyst discharge. Biol. Bull. *167*, 139–158.
14. Lange, R., Plickert, G., and Müller, W.A. (1989). Histoincompatibility in a low invertebrate, *Hydractinia echinata*: analysis of the mechanism of rejection. J. Exp. Zool. *249*, 284–292.
15. Ivker, F.B. (1972). A hierarchy of histo-incompatibility in *Hydractinia echinata*. Biol. Bull *143*, 162–174.
16. Hauenschild, C. (1956). Über die Vererbung einer Gewebeverträglichkeitseigenschaft bei dem Hydroidpolypen *Hydractinia echinata*. Z Naturforsch Pt B *11*, 132–138.
17. Hauenschild, C. (1954). Genetische und entwichlungphysiologische Untersuchungen über Intersexualität und Gewebeverträlichkeit bei *Hydractinia echinata* Flem. Rouxs Arch. Dev. Biol. *147*, 1–41.
18. Grosberg, R.K., Levitan, D.R., and Cameron, B.B. (1996). Evolutionary genetics of allorecognition in the colonial hydroid *Hydractinia symbiolongicarpus*. Evolution Int. J. Org. Evolution *50*, 2221–2240.
19. Mokady, O., and Buss, L.W. (1996). Transmission genetics of allorecognition in *Hydractinia symbiolongicarpus* (Cnidaria:Hydrozoa). Genetics *143*, 823–827.

20. Cadavid, L.F., Powell, A.E., Nicotra, M.L., Moreno, M., and Buss, L.W. (2004). An invertebrate histocompatibility complex. Genetics *167*, 357–365.
21. Powell, A.E., Nicotra, M.L., Moreno, M.A., Lakkis, F.G., Dellaporta, S.L., and Buss, L.W. (2007). Differential effect of allorecognition loci on phenotype in *Hydractinia symbiolongicarpus* (Cnidaria: Hydrozoa). Genetics *177*, 2101–2107.
22. Barrow, A.D., and Trowsdale, J. (2006). You say ITAM and I say ITIM, let's call the whole thing off: the ambiguity of immunoreceptor signalling. Eur. J. Immunol. *36*, 1646–1653.
23. Cosman, D., Fanger, N., and Borges, L. (1999). Human cytomegalovirus, MHC class I and inhibitory signalling receptors: more questions than answers. Immunol. Rev. *168*, 177–185.
24. Harpaz, Y., and Chothia, C. (1994). Many of the immunoglobulin superfamily domains in cell adhesion molecules and surface receptors belong to a new structural set which is close to that containing variable domains. J. Mol. Biol. *238*, 528–539.
25. Nyholm, S.V., Passegue, E., Ludington, W.B., Voskoboynik, A., Mitchel, K., Weissman, I.L., and De Tomaso, A.W. (2006). *fester*, a candidate allorecognition receptor from a primitive chordate. Immunity *25*, 163–173.
26. Poudyal, M., Rosa, S., Powell, A.E., Moreno, M., Dellaporta, S.L., Buss, L.W., and Lakkis, F.G. (2007). Embryonic chimerism does not induce tolerance in an invertebrate model organism. Proc. Natl. Acad. Sci. USA *104*, 4559–4564.
27. Lange, R.G., Dick, M.H., and Müller, W.A. (1992). Specificity and early ontogeny of historecognition in the hydroid *Hydractinia*. J. Exp. Zool. *262*, 307–316.
28. Yund, P.O., Cunningham, C.W., and Buss, L.W. (1987). Recruitment and postrecruitment interactions in a colonial hydroid. Ecology *68*, 971–982.
29. Nicotra, M.L., and Buss, L.W. (2005). A test for larval kin aggregations. Biol. Bull. *208*, 157–158.
30. Pond, S.L.K., and Frost, S.D.W. (2005). Datamonkey: rapid detection of selective pressure on individual sites of codon alignments. Bioinformatics *21*, 2531–2533.
31. Wright, S. (1939). The distribution of self-sterility alleles in populations. Genetics *24*, 538–552.
32. Cannon, J.P., Haire, R.N., and Litman, G.W. (2002). Identification of diversified genes that contain immunoglobulin-like variable regions in a protochordate. Nat. Immunol. *3*, 1200–1207.
33. Pancer, Z., Amemiya, C.T., Ehrhardt, G.R.A., Ceitlin, J., Larry Gartland, G., and Cooper, M.D. (2004). Somatic diversification of variable lymphocyte receptors in the agnathan sea lamprey. Nature *430*, 174–180.
34. Zhang, S.M., Adema, C.M., Kepler, T.B., and Loker, E.S. (2004). Diversification of Ig superfamily genes in an invertebrate. Science *305*, 251–254.
35. Marra, M.A., Kucaba, T.A., Dietrich, N.L., Green, E.D., Brownstein, B., Wilson, R.K., McDonald, K.M., Hillier, L.W., McPherson, J.D., and Waterston, R.H. (1997). High throughput fingerprint analysis of large-insert clones. Genome Res. *7*, 1072–1084.
36. Loytynoja, A., and Goldman, N. (2008). Phylogeny-aware gap placement prevents errors in sequence alignment and evolutionary analysis. Science *320*, 1632–1635.
37. Buss, L.W., and Blackstone, N.W. (1991). An experimental exploration of Waddington epigenetic landscape. Philos. Trans. R. Soc. London B *332*, 49–58.